



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/00	A2	(11) International Publication Number: WO 99/42821 (43) International Publication Date: 26 August 1999 (26.08.99)															
<p>(21) International Application Number: PCT/GB99/00506</p> <p>(22) International Filing Date: 18 February 1999 (18.02.99)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>9803446.5</td> <td>18 February 1998 (18.02.98)</td> <td>GB</td> </tr> <tr> <td>9803447.3</td> <td>18 February 1998 (18.02.98)</td> <td>GB</td> </tr> <tr> <td>9812151.0</td> <td>5 June 1998 (05.06.98)</td> <td>GB</td> </tr> <tr> <td>9814545.1</td> <td>3 July 1998 (03.07.98)</td> <td>GB</td> </tr> <tr> <td>9903035.5</td> <td>10 February 1999 (10.02.99)</td> <td>GB</td> </tr> </table> <p>(71) Applicant (for all designated States except US): THERYTE LIMITED [GB/GB]; 5 Castle Street, Liverpool L2 4XE (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): WARENIUS, Hilmar, Meek [GB/GB]; 14 Delavor Road, Heswall, Wirral L60 4RN (GB). SEABRA, Laurence, Anthony [GB/GB]; 12 Richmond Way, Heswall, Wirral L61 6XH (GB).</p> <p>(74) Agents: DANIELS, Jeffrey, Nicholas et al.; Page White & Farrer, 54 Doughty Street, London WC1N 2LS (GB).</p>		9803446.5	18 February 1998 (18.02.98)	GB	9803447.3	18 February 1998 (18.02.98)	GB	9812151.0	5 June 1998 (05.06.98)	GB	9814545.1	3 July 1998 (03.07.98)	GB	9903035.5	10 February 1999 (10.02.99)	GB	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
9803446.5	18 February 1998 (18.02.98)	GB															
9803447.3	18 February 1998 (18.02.98)	GB															
9812151.0	5 June 1998 (05.06.98)	GB															
9814545.1	3 July 1998 (03.07.98)	GB															
9903035.5	10 February 1999 (10.02.99)	GB															
<p>(54) Title: TREATING CANCER</p> <p>(57) Abstract</p> <p>Provided is a method for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising testing a sample comprising cells or an extract therefrom for the co-elevation of CDK1 and CDK4. Also provided is a kit for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising: (i) a means for testing for the elevation of CDK1; and (ii) a means for testing for the elevation of CDK4.</p>																	

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TREATING CANCER

The present application concerns methods of diagnosing cancer. In particular the application concerns a method for diagnosing cancer by measuring the levels of two proteins present in suspected cancer cells.

The hallmark of cancer is a capacity for unlimited, autonomous cellular proliferation. The discovery of a means of selective inhibition of proliferation in cancer cells without concomitant damage to the proliferative capacity of normal cells, could potentially provide new ways to halt the growth of tumours irrespective of their degree of differentiation, invasion or metastasis. It is interesting to understand how cancer cells divide and whether this may differ from normal cell division. Most treatments for cancer are broadly cytotoxic, generally targeting proliferating cells. Normal tissues also proliferate, however, and so are also damaged by cytotoxic agents. Identification of cancer specific markers that permit targeting or that provide specific targets for drug development would allow the development of treatments that are more specifically toxic to tumour tissues thus reducing the debilitating effects of chemotherapy..

The effect of the expression of single genes alone on the response of human cancer cell lines to treatment with cytotoxic drugs such as CDDP has been studied in human *in vitro* cell lines because these present a model system relevant to the response of human cancer in the clinic. In particular, they exhibit the range of sensitivities to cytotoxic drugs and ionising radiation usually encountered in the clinic. Discoveries in human *in vitro* cell lines, therefore, have a strong possibility of being able to be translated into clinically useful tests for how well cancers may be expected to respond to treatment.

The progress of cells through the cell cycle is governed by holoenzymes formed by a combination of proteins called cyclins, whose levels fluctuate throughout the cell cycle, and cyclin dependent kinases (CDKs) which become active when they join with cyclins. The cyclin/CDK complexes can be inhibited by proteins termed cyclin dependent kinase inhibitors (CDKIs) which include the protein p21 WAF1/CIP1 (p21).

The protein products of the cyclin D1 and B1 genes and their respective cyclin-dependent kinase partners CDK4 and CDK1 have been studied. Cyclin D1 and CDK4 control the progress of cells through the cell cycle checkpoint between G1 and S-phase (the phase of DNA synthesis). Cyclin B1 and CDK1 control the cell cycle checkpoint just before mitosis. The expression of cyclin D1 protein in a series of 16 human cancer cell lines has been shown to be related to their intrinsic resistance to the cytotoxic drug CDDP (Warenus *et al.*, 1996). Cyclin D1 protein levels, however, showed no relationship with radiosensitivity, another treatment modality. The relationship between cyclin D1 and CDDP resistance is not, however, strong enough on its own to provide the basis of clinically useful predictive assays.

The present invention aims to solve the problems associated with the above prior art and to provide an improved method for the diagnosis of cancer.

Accordingly, this invention describes methods of diagnosing a cancerous state by contemporaneously measuring the properties of two or more cancer-related genes. This application deals with measuring the levels of the protein product of the CDK1 gene and the protein product of the CDK4 gene, preferably whilst also determining the mutational status of p53.

Specifically this invention provides a method for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising testing a sample comprising cells or an extract therefrom for the co-elevation of CDK1 and CDK4.

The co-elevation of CDK1 and CDK4 can be either elevation in their expression or their protein levels. The elevation of expression of CDK1 and CDK4, or the elevation of CDK1 and CDK4 protein levels can be measured by any appropriate method, e.g. Western blotting. The point at which it is considered that the level is elevated or that the expression is elevated (or over-expression) is clear to the skilled person in this field, according to general teaching from the literature regarding usual levels of CDK1 and CDK4 in human cell lines (see Cancer Research, 1994, Nov 15; 54(22), 5804-7; Cancer Research, 1993, Nov. 15; 53(22), 5535-41;

and British Journal of Cancer, 1995, Jun.; 71(6), 1231-6). This point can be determined according to the judgement of the individual carrying out the present method, depending on the particular cells and patient involved.

The invention also provides a kit for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising:

- (i) a means for testing for the elevation of CDK1; and
- (ii) a means for testing for the elevation of CDK4.

Furthermore this invention provides a novel, complex, target for drug screening which might lead to drugs that are more specifically toxic to cancer tissues with the features disclosed in this invention.

This invention specifically deals with measuring the levels of CDK1 protein and levels of CDK4 protein, in cells. The p53 mutational status of the cells has preferably been identified, e.g. by DNA sequencing. High levels of CDK1 and CDK4 are found in human cancer cells, especially those bearing p53 mutations.

Modulation of the relationship between CDK1 and CDK4 in p53 Mutant Human cells

The above are unusual findings that do not fit with what is widely known about CDKs at the present time. Disruption of the CDK1/CDK4 relationship in p53 mutant cells can be identified as a new target for anticancer therapy. In addition because both the CDK1/CDK4 co-elevation and p53 mutation are confined to cancer cells and appear to be inter-related, they form in combination a complex target that is likely to prove the most specific one for cancer therapy that has so far been discovered.

The invention will now be described in further detail by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows the correlation between CDK1 and CDK4 levels in human cancer cell lines as well as the corresponding levels in normal cells such as human keratinocytes and human fibroblasts;

Figure 2 shows the relative lack of correlation between cyclin D and cyclin B (the respective partners of CDK4 and CDK1) levels in human cancer cell lines;

Figure 3 shows the range of values for CDK1 levels found from several Western immunoblot runs carried out on specific human *in vitro* cell lines (the standard errors are also shown);

Figure 4 shows the corresponding data to Figure 3, obtained in respect of CDK4;

Figure 5 shows the correlation between CDK1 and CDK4 levels in p53 mutant human cancer cell lines; and

Figure 6 shows the corresponding correlation to Figure 5, obtained in respect of wild-type p53 human cell lines.

A Dual Parameter Test for Cancer using CDK1 and CDK4 Protein Expression

A clinical test for cancer is provided based on the measurement of CDK1 protein expression levels, CDK4 protein expression levels and detection of mutations in the p53 gene. In a research environment CDK protein levels are typically measured by Western blotting or by immunocytochemistry but for diagnostic purposes cheaper and more rapid methods are more preferable.

The determination of the mutational status of p53 can be effected by sequencing the genomic locus bearing the gene from the patient or by sequencing the expressed mRNA after conversion to cDNA. Various nucleic acid sequencing methodologies are available at present, all of which are appropriate for use with this diagnostic assay. The most widely used method would be based on incorporation of terminating nucleotides into polymerase generated copies of a template, using the method of Sanger *et al*, 1977. Many alternatives have arisen recently

including adaptor sequencing (PCT/US95/12678), ligation based sequencing (PCT/US96/05245), sequencing by hybridisation (A.D. Mirzabekov, TIBTech 12: 27-32, 1994) to list a few. Various methods for testing for specific mutations exist and are well known in the art, such as the TaqMan assay, oligonucleotide ligase assays, single strand conformational polymorphisms and assays based on hybridisation of template nucleic acids to oligonucleotide arrays.

Because CDK1 and CDK4 are not under direct transcriptional control, it is unlikely that mRNA levels for CDK1 and CDK4 will follow the same pattern as their proteins. This means that determination of the mRNA levels of these genes is not sufficient for the purposes of this test.

Immunocytochemistry

In a preferred embodiment of this invention, CDK1 and CDK4 protein levels could be measured by immunocytochemistry using confocal laser fluorescence microscopy to detect antibody binding. Preferably a scanning system would be used such as those described in PCT/US91/09217, PCT/NL/00081 and PCT/US95/01886. An antibody against CDK1 (such as the Mouse monoclonal sc-54 from Santa Cruz Biotechnology, CA) would be labelled with one dye, an antibody against CDK4 (such as the purified rabbit polyclonal sc-260 from Santa Cruz Biotechnology, CA) would be labelled with a second dye whilst a third DNA binding dye could be used to select for aneuploid cells. DNA binding dyes such as Hoechst 33258 dye, which binds AT-rich DNA or Chromomycin A₃, which binds GC-rich DNA, would be appropriate. The intensity of fluorescence from these dyes would provide an indication of the expression level of the genes. A diagnostic test may comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Preparing the biopsy material for microscopy which includes the steps of:
 - ♦ Labelling the biopsy material with the above fluorescently labelled antibody probes against CDK1 and CDK4. The biopsy material may also optionally be labelled with antibody probes against p53 mutant proteins and with a DNA binding dye.
 - ♦ Separating the labelled cells from unbound labelled probes.

- Placing the labelled biopsy material in a scanning confocal microscope to count cells that:
 - ◆ Over-express or show elevation of CDK1, i.e. are labelled with at least a threshold quantity of antibody against CDK1.
 - ◆ Over-express or show elevation of CDK4, i.e. are labelled with at least the threshold quantity of antibody against CDK4.
 - ◆ Optionally, express mutant forms of p53, i.e. are labelled with at least the threshold quantity of antibodies against p53 mutants. Alternatively, p53 mutational status might be determined by analysis of the mRNA or genomic DNA as discussed above.
 - ◆ Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Fluorescence Activated Cell Sorting

One embodiment of the diagnostic test could exploit Fluorescence Activated Cell Sorting (FACS). A FACS instrument separates cells in a suspension in a manner dependant on the cells being labelled with a fluorescent marker. A typical FACS device operates as follows. Cells in a suspension travelling in single file are passed through a vibrating nozzle which causes the formation of droplets containing a single cell or none at all. The droplets pass through a laser beam. Fluorescence from each individual cell in its droplet, excited by a laser, is measured. After the detector the stream of cells in suspension pass through an electrostatic collar which gives the droplets a surface charge. The cell carrying droplets are given a positive or negative charge. If the drop contains a cell that fluoresces with an intensity above a particular threshold, the drop gets a charge of one polarity. Unlabelled cells get a charge of the opposite polarity. The charged droplets are then deflected by an electric field and, depending on their surface charge, are directed into separate containers and are counted. Droplets that contain more than one cell scatter light more than individual cells. This is readily detected and so these are left uncharged and enter a third disposal container.

Multi-channel fluorescent detection devices have been constructed that can separate cells on the basis of labelling with multiple different fluorescent labels. These have multiple lasers which can excite fluorescence at different frequencies and the detector will detect different emission frequencies. Using this technique a test could be carried out using a multi parameter

array on a flow cytometer without the need for sorting. A three label system would be appropriate for this test. An antibody against CDK1 would be labelled with one dye, an antibody against CDK4 would be labelled with a second dye whilst a third, DNA binding dye can be used to select for aneuploid cells. DNA binding dyes such as Hoechst 33258 dye, which binds AT-rich DNA, or Chromomycin A₃, which binds GC-rich DNA, are appropriate. Additionally, a number of antibodies are commercially available which can detect some of the mutant forms of p53. Antibodies such as these may be labelled with a fourth dye. The intensity of fluorescence from these dyes gives an indication of the expression levels of the two proteins and indicates the chromosomal status of labelled cells passing the detector. A minimum level of fluorescence intensity from each dye present in an individual cell may be required to classify a cell as being cancerous. At present not all mutant forms of p53 can be detected using antibodies, although antibodies exist against a number of known mutant forms of the p53 protein. A diagnostic test may comprise the steps of:

- Extracting a biopsy of the tumour from a patient.
- Optionally micro-dissecting that material to separate normal tissue from tumour material.
- Disrupting intracellular adhesion to form a single cell suspension.
- Labelling the suspended cells with the above fluorescently labelled probes against CDK1 and CDK4. The biopsy material may also, optionally be labelled with antibody probes against p53 mutant proteins and with a DNA binding dye.
- Separating the labelled cells from unbound labelled probes.
- Passing the labelled cell suspension through a FACS device to count cells that:
 - ◆ Over-express or show elevation of CDK1, i.e. are labelled with at least a threshold quantity of antibody against CDK1.
 - ◆ Over-express or show elevation of CDK4, i.e. are labelled with at least the threshold quantity of antibody against CDK4.
 - ◆ Optionally, express mutant forms of p53, i.e. are labelled with at least the threshold quantity of antibodies against p53 mutants. Alternatively, p53 mutational status may be determined by analysis of the mRNA or genomic DNA as discussed above.
 - ◆ Optionally, have chromosomal amplifications as detected by the intensity of fluorescence from DNA binding fluorescent dyes.

Example 1

Human *in vitro* cell lines of different histological origin which exhibit a range of intrinsic sensitivity to cytotoxic drugs as measured by clonogenic cell survival assays, have been shown to provide appropriate models of clinical tumours, particularly in their responses to chemotherapy. In particular, these cell lines exhibit the range of sensitivities to cytotoxic drugs and ionising radiation usually encountered in the clinic. These human *in vitro* cancer cell lines are now widely recognised as relevant models for the clinical response of tumours to chemotherapy. It is therefore possible to identify genes from these cancer models whose expression and/or mutational status is tumour specific, which are also features of real clinical tumours. Discoveries in human *in vitro* cell lines, such as those leading to this invention, therefore, have a strong possibility of being able to be translated into meaningful targets for drug discovery programs.

A body of work has been carried out analysing the expression of a number of genes that have been implicated in the cancer disease process. In contrast to any of the other genes controlling cell division that were examined, CDK1 and CDK4 appeared to be consistently co-elevated in a large series of human cancer cell lines and extracts from clinical colon cancer. Without being limited by theory, it is hypothesised that for cancer cells to be able to continue to divide successfully it may be necessary a) for CDK1 and CDK4 to retain their normal functions and b) for the elevated levels of these two proteins in human cancer to be related in some way, the mechanism of which is unclear.

To investigate the above hypothesis DNA sequencing was carried out on all the exons of both CDK1 and CDK4 in the 20 human cell lines in which a strong relationship between the expression of the two proteins was detected. There were no mutations in the exons of the CDK1 and CDK4 genes in the 20 human *in vitro* cell lines that were sequenced. This finding is very surprising, because cancer cells are well known to progressively accumulate mutations in the critical genes controlling cell division and this is held by many scientific practitioners in this field to be most marked in human *in vitro* cell lines such as those investigated.

Materials and Methods

Cell lines and clonogenic cell survival assays

The growth characteristics clonogenic assay procedures of the human *in vitro* cell lines used in this analysis have already been reported (Warenius *et al* 1994). The cell lines are listed, with their histological classification in Table 1. All are well established; many having been growing *in vitro* for several years. Cell lines were either donations or purchased by our laboratories. On receipt all were grown for 5 passages to provide sufficient cells for batch storage in liquid nitrogen. During this period contamination was excluded by at least one passage in antibiotic free medium and mycoplasma testing was carried out on all lines. For clonogenic assays, cells were taken from a designated primary liquid nitrogen batch and grown for 3-6 passages until there were sufficient well-growing cells. Further batches from these cells were frozen in liquid nitrogen. Cells were routinely maintained in DMEM medium except RT112 and H322, which were grown in RPMI1640 and MGHU-1 which were grown in Ham's F12 medium. All lines were supplemented with 10% heat-inactivated foetal calf serum (HIFCS).

Table 1 - p53 mutational status

	Cell Line	cDNA sequence	Amino-acid change	p53 protein
I407	Embryonic intest. epith.	Normal	none	Wild-type
HEP 2	Squamous carcin. larynx	Normal	none	Wild-type
MGHU 1	Transit. carcinoma bladder	Normal	none	Wild-type
HRT 18	Adenocarcinoma rectum	Normal	none	Wild-type
2780	Ovarian carcinoma	Normal	none	Wild-type
OAW 42	Ovarian carcinoma	CGA-CGG codon 213	none	Wild-type
HT 29/5	Adenocarcinoma colon	CGT-CAT codon 273	Arg-His	Mutant
COLO 320	Adenocarcinoma colon	CGG-TGG codon 245	Arg-Tryp	Mutant
H 322	Small cell carcinoma lung	CGG-TGG codon 245	Arg-Tryp	Mutant
H 417	Small cell carcinoma lung	GAG-TAG codon 298	Glu-Stop	Truncated
RPMI 7951	Melanoma	TCA-TTA codon 166	Ser-Stop	Truncated
RT 112	Transit. carcinoma bladder	CCG-CAG codon 248	Arg-Gly	Mutant
MOR	Adenocarcinoma lung	C deletion codon 152	Frameshift 178aa	Truncated
MEL 2	Melanoma	CGC-AGC codon 245	Gly-Ser	Mutant

Identification of mutations in the p53 gene by PCR and DNA sequencing

Material for PCR and DNA sequencing of p53 was obtained from the same liquid nitrogen batches used to provide cells for clonogenic cell survival data. Cells were grown for up to three passages prior to being subjected to the following procedures:

Nucleic Acid Isolation

RNA and genomic DNA were prepared from the cell lines described here by the guanidinium isothiocyanate CsCl gradient method (Chirgwin *et al*, 1979, Barraclough *et al*, 1987). Briefly, the cells were collected in ice-cold phosphate-buffered saline (PBS) and homogenised in guanidinium isothiocyanate buffer (4M guanidinium isothiocyanate, 50mM Tris pH 7.5, 25mM EDTA pH 8.0, 0.5% (w/v) sodium lauryl sarcosine and 8% (v/v) 2-mercaptoethanol added just prior to use. The homogenate was cleared by centrifugation at 8,000 rpm for 10 mins at 4°C (SS34 rotor, Sorvall RC-5B centrifuge) and the RNA pelleted by centrifugation of the homogenate through a cushion of 5.7M caesium chloride/0.1M EDTA at 32,000 rpm for 20hr at 20°C (TST 41.14 rotor, Kontron Centrikon T20 60 ultracentrifuge). The pellet of RNA was redissolved in 0.1% (w/v) SDS and precipitated with ethanol overnight at -20°C before quantitation.

Polymerase Chain Reaction, cDNA synthesis and nucleotide sequencing

PCR (for exons 2-8 and for exons 9-11) was performed on DNA and RNA extracted from the human carcinoma cell lines. Each exon was then examined by DNA sequencing. PCR Primers were designed flanking each exon and synthesised on an Applied Biosystems 381A DNA synthesiser. Each exon was amplified separately with the exceptions of exons 2 and 3 which were amplified as a unit, and exons 9, 10 and 11 which were amplified together by reverse transcription polymerase chain reaction (RT-PCR). The following primers were used:

Exon 2/3 sense 5'-CCC ACT TTT CCT CTT GCA GC-3'

Exon 2/3 antisense 5'-AGC CCA ACC CTT GTC CTT AC-3'

Exon 4 sense 5'-CTG CTC TTT TCA CCC ATC TA-3'

Exon 4 antisense 5'-GCA TTG AAG TCT CAT GGA AG-3'

Exon 5 sense 5'-TGT TCA CTT GTG CCC TGA CT-3'

Exon 5 antisense 5'-CAG CCC TGT CGT CTC TCC AG-3'

Exon 6 sense 5'-GCC TCT GAT TCC TCA CTG AT-3'

Exon 6 antisense 5'-TTA ACC CCT CCT CCC AGA GA-3'

Exon 7 sense 5'-ACT GGC CTC ATC TTG GGC CT-3'

Exon 7 antisense 5'-TGT GCA GGG TGG CAA GTG GC-3'

Exon 8 sense 5'-T ATC CTG AGT AGT GG-3'

Exon 8 antisense 5'-T GCT TGC TTA CCT CG-3'

Exon 9/10/11 sense 5'-AGA AAG GGG AGC CTC ACC AC-3'

Exon 9/10/11 antisense 5'-CTG ACG CAC ACC TAT TGC AA-3'

Genomic DNA was digested with EcoR1 and precipitated with ethanol and resuspended in 50µl of water (Sigma) before being subjected to PCR amplification. The DNA (1µg) was amplified in 50µl PCR reactions containing 20 pmoles of each primer. A 'hot start' PCR protocol was used with the dNTP's and Taq enzyme initially separated from the rest of the reaction components on a wax cushion. The reactions were placed in a pre-heated PCR block at 95°C for 2 minutes before undergoing thirty cycles of denaturation (30s at 95°C), annealing (30s at 60°C for exons 2-3, 4 and 6; 65°C for exons 5 and 8; 67°C for exon 7; and 68°C for exon 9-11) and extension (1 min at 72°C). The PCR products were checked on a 0.8% (w/v) agarose gel before being purified using a Wizard minicolumn (Promega), and used directly in sequencing reactions.

cDNA synthesis and RTPCR

Complementary DNA was synthesised from approximately 5µg of total RNA using oligo (dT) as a primer. Total RNA (5µg), human placental ribonuclease inhibitor (HPRI) 20U and 1µg oligo (dT) were heated at 70°C for 10 minutes, chilled on ice, added to 1x first strand buffer (50mM Tris-HCl, pH 8.3, 75mM potassium chloride and 3mM magnesium chloride), 0.01M DTT, dNTPs (0.5mM for each deoxyribonucleoside triphosphate), 400U of Superscript Reverse Transcriptase (Gibco) and incubated at 37°C for 1 hour. PCR for exons 9 to 11 was carried out using 5µl of the above incubation in a 50µl of PCR reaction as described in the previous section.

Nucleotide Sequencing

Sequencing primers (10 pmoles) were radioactively labelled at their 5' ends with ^{32}P -ATP (45 μCi) at 37°C for 30 min in a reaction containing T4 Polynucleotide Kinase (PNK) (9.7U, Pharmacia) and 1x T4 PNK buffer (10mM Tris-acetate, 10mM magnesium acetate and 50mM potassium acetate). The primers used were identical to those employed in the PCR reactions except for exon 5 for which a separate sense sequencing primer was designed as follows: 5'-TAC TCC CCT GCC CTC-3'. Sequencing was carried out by the dideoxynucleotide enzymatic method (Sanger *et al*, 1977), using the fmol DNA Sequencing System (Promega). Any putative sequence mutations identified were confirmed by additional sequencing of the exon in the antisense direction as well as by carrying out a repeat PCR and sequencing of the cell line.

Results

Mutations were found in mRNA expressed from the p53 gene in a number of cell lines (see Table 1). The mutations identified in the cell lines described here were in exons 5-8 which are known to contain the majority of p53 mutations (Hollstein *et al*, 1991). All these mutations have been previously described apart from the nonsense mutation identified in codon 166 of the RPMI7951 line. This along with the G to T transversion in codon 298 of H417 did not lie within the most highly conserved region of the p53 gene. In the OAW42 ovarian carcinoma cell line the single base missense mutation from CGA to CGG was silent, so that the mutant triplet still coded for the same amino acid (Arg) as is present in wild-type p53 (wtp53) protein. A normal p53 protein was thus expressed in half of the cell lines. The mRNA of the other half of the cell lines coded for abnormal p53 protein. RPMI7951 and H417 possessed stop mutations resulting in 165 and 297 amino acid truncated proteins respectively. COLO320 and H322 independently exhibited a missense G:C to A:T mutation at the same site resulting in an amino-acid substitution from Arg to Tryp. RT112 and HT29/5 also had mutations coding for changes in Arg (to Gly and His respectively). COLO320, H322 and RT112 were homozygous for p53 mutations. The other mutant lines showed evidence of retention of heterozygosity. HT29/5 and RPMI7951 both expressed small amounts of wild-type p53 mRNA though H417 expressed relatively high levels.

Western Blotting for CDK1 and CDK4

Two independent Western blottings with lysates for each cell line loaded in pairs on each gel were carried out. 10^7 cells were grown in 162 cm² tissue culture flasks (Costar Ltd., High Wycombe, Bucks until they were pre-confluent but still growing exponentially. Cells were then removed by trypsinisation, resuspended in complete medium + 10% FCS and washed 3 times by serial centrifugation and resuspension in PBS without serum. $1-3 \times 10^8$ viable cells were then pelleted by centrifugation and resuspended at 3×10^7 cells per ml of lysate buffer (Stock solution: 10% SDS 10ml., 0.5M Tris pH 6.8, glycerol 10 ml., Double distilled water 62 ml. To 10 ml. of stock solution were added 100 ml of 10 mM Leupeptin + 10 ml 100 mM PMSF). Protein estimations were performed and the final concentration of the lysates adjusted to 300 mg total cellular protein per 100 ml. To measure CDK1 and CDK4 proteins, 150µg of total cellular protein in 50 µl of lysate buffer was added per lane well to a 7.5% Laemmli separating gel and electrophoresis carried out at 16°C using 60V over 16 hours and a constant current of 500mA. Blots were transferred to nitrocellulose at 22°C over 16 hours using to a semi-dry blotting apparatus (Biorad, Richmond, CA). To determine CDK1 protein expression levels the blot was incubated with the sc-054 mouse monoclonal antibody to human CDK1 (Santa Cruz Biotechnology, CA) and then incubated with rabbit anti-mouse conjugated antibodies (Dako UK) at 1/1000 and developed in alkaline phosphatase buffer containing Nitroblue Tetrazolium and 5-Bromo-4-Chloro-3-Indoyl Phosphate, (Sigma, Poole, Dorset, UK) (50mg/ml in dimethylformamide). To determine CDK4 protein expression levels the blot was incubated with the sc-260 rabbit poly-clonal antibody to human CDK4 (Santa Cruz Biotechnology, CA) and then incubated with rabbit anti-mouse conjugated antibodies (Dako UK) at 1/1000 and developed in alkaline phosphatase buffer containing Nitroblue Tetrazolium and 5-Bromo-4-Chloro-3-Indoyl Phosphate, (Sigma, Poole, Dorset, UK) (50mg/ml in dimethylformamide).

Quantitation of the protein product of the CDK1 and CDK4 genes was carried out by measurement of optical density on a Shimadzu scanning densitometer with tungsten light and expressed as O.D. units per 150 µg of total cellular protein. Titration curves obtained by loading different amounts of total cellular protein have previously shown that linear relationships for optical density (O.D.) could be obtained over the range found for CDK1 and

CDK4 protein across the cell lines. In order to compare different CDK1 and CDK4 protein levels between the cell lines, the mean O.D. value for all the lines was calculated and the relative O.D. for CDK1 and CDK4 protein in each individual cell line was normalised to the mean O.D. and multiplied by an arbitrary value of 5.0.

The cell lines investigated are shown in Table 2 below.

Table 2

Cell Line	Histology
2780	Ovarian carcinoma
A431	Squamous carcinoma vulva
A549	Adenocarcinoma lung
AT5BIVA	Ataxia telangiectasia transformed fibroblast
COR L23	Large cell lung carcinoma
G361	Melanoma
H322	Small cell carcinoma lung
HEP2	Squamous carcinoma larynx
HRT18	Adenocarcinoma rectum
HT29/5	Adenocarcinoma colon
HX142	Neuroblastoma
HX34	Skin melanoma
I407	Embryonic intestinal epithelium
KB	Oral epidermoid carcinoma
MEL2	Melanoma
MEL3	Melanoma
MGHU-1	Transit cell carcinoma bladder
MOR	Adenocarcinoma lung
OAW42	Ovarian carcinoma
RT112	Transit cell carcinoma bladder

The results are shown in Figures 3 and 4.

A strong correlation between the expression of the CDK1 and CDK4 in a series of human *in vitro* cancer cell lines is observed. Using DNA sequencing data for p53 mutational status for

a number of these lines, it is further observed that the CDK1/CDK4 relationship is particularly marked in cell lines with p53 mutations (see Figure 5 and Figure 6)

Also, surprisingly, the correlation seen between the protein expression for these cell lines was not seen for the relevant mRNAs. Nor was there a reciprocal correlation between the protein expression for one gene and the mRNA for the other. These results suggest that the co-elevation of CDK1 and CDK4 proteins is not at the level of transcriptional control but may be at a translational or post-translational stage. That is to say the proteins themselves are influencing each other in some way or are both influenced by an as yet unknown factor.

The findings concerning the co-relationships of CDK1 and CDK4 proteins in human *in vitro* cell lines and clinical colon cancers are supported by transfection studies into RAMA37, a rat normal myoepithelial cell line. Pilot studies with RAMA37 suggest that over-expression of CDK4 protein as a result of successful transfection of CDK4 under unconditional promoter control is accompanied by a concomitant elevation in the constitutive expression of CDK1.

References

- Barraclough *et al*, J. Cell Physiology **131**: 393 - 401, 1987.
- Chirgwin *et al*, Biochemistry **18**: 5294 - 5299, 1979.
- Maskos and Southern, Nucleic Acids Research **21**, 2269 - 2270, 1993.
- Pease *et al*. Proc. Natl. Acad. Sci. USA. **91**, 5022 - 5026, 1994.
- Sanger *et al*, Proc. Natl. Acad. Sci. USA **74**: 5463 - 5467, 1977.
- Southern *et al*, Nucleic Acids Research **22**, 1368 - 1373, 1994.
- Warenius *et al.*, Int.J.Cancer. **67**: 224 - 231, 1996.
- Bristow *et al.*, Oncogene **9**: 1527 - 1536, 1994.
- Bristow *et al.*, Radiotherapy and Oncology **40**: 197 - 223, 1996.
- P. W. G. Browning, "Proto-oncogene expression and intrinsic radiosensitivity, PhD Thesis, University of Liverpool, 1997.
- Deacon *et al*, Radiotherapy and Oncology **2**, 317 - 323, 1984.
- Fan *et al*, Cancer Res. **54**: 5824 - 5830, 1994.
- Fertil & Malaise, Int. J. Radiat. Oncol. Biol. Phys. **7**: 621 - 629, 1981.
- FitzGerald *et al*, Radiat. Res. **122**: 44 - 52, 1990.
- Hollstein *et al*, Science **253**: 49 - 53, 1991.
- Iliakis *et al.*, Cancer Res. **50**: 6575 - 6579, 1990.
- Kasid *et al.*, Cancer Res. **49**: 3396 - 3400, 1989.
- Kastan *et al*, Cancer Res. **51**: 6304 - 6311, 1991.
- Kawashima *et al.*, Int. J. Cancer **61**: 76 - 79, 1995.
- Kelland *et al*, Radiat. Res. **116**: 526 - 538, 1988
- Lee and Bernstein, Proc. Natl. Acad. Sci. USA **90**: 5742 - 5746, 1993.
- McIlwrath *et al.*, Cancer Res. **54**: 3718 - 3722, 1994.
- McKenna *et al.*, Cancer Res. **50**: 97 - 102, 1990.
- McKenna *et al.*, Radiat. Res. **125**: 283 - 287, 1991
- Nunez *et al*, Br. J. Cancer **71**: 311 - 316, 1995
- Pardo *et al.*, Radiat. Res. **140**: 180 - 185, 1994
- Pirollo *et al.*, Int. J. Radiat. Biol. **55**: 783 - 796, 1989
- Pirollo *et al.*, Radiat. Res. **135**: 234 - 243, 1993.
- Powell & McMillan, Int. J. Rad. Oncol. Biol. Phys., **29**: 1035 - 1040, 1994.

- Radford, Int. J. Radiat. Biol. 66: 557 - 560, 1994.
- Sanger *et al.*, Proc. Natl. Acad. Sci. USA 74: 5463 - 5467, 1977.
- Schwartz *et al.*, Int. J. Radiat. Biol. 59: 1314 - 1352, 1991.
- Shimm *et al.*, Radiat. Res. 129: 149 - 156, 1992.
- Siles *et al.*, Br. J. Cancer 73: 581 - 588, 1996.
- Su & Little, Int. J. Radiat. Biol. 62: 461 - 468, 1992.
- Su & Little, Radiat. Res. 133: 73 - 79, 1993.
- Suzuki *et al.*, Radiat. Res. 129: 157 - 162, 1992.
- Warenius *et al.*, Eur. J. Cancer 30, 369 - 375, 1994.
- Warenius *et al.*, Rad. Research 146, 485 - 493, 1996.
- Whitaker *et al.*, Int. J. Radiat. Biol. 67: 7 - 18, 1995.
- Xia *et al.*, Cancer Res. 55: 12 - 15, 1995.
- Zhen *et al.*, Mut. Res. 346, 85 - 92, 1995.

CLAIMS:

1. A method for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising testing a sample comprising cells or an extract therefrom for the co-elevation of CDK1 and CDK4.
2. A method according to claim 1, wherein the sample is extracted from the subject.
3. A method according to claim 1 or claim 2, wherein the testing comprises contacting the sample with a labelled antibody against CDK1 and/or a labelled antibody against CDK4.
4. A method according to claim 3, wherein the antibody against CDK1 is sc-54 (Santa Cruz Biotechnology Inc., CA) and/or the antibody against CDK4 is sc-260 (Santa Cruz Biotechnology Inc., CA)
5. A method according to any preceding claim, wherein the cells are mutant p53 cells.
6. A method according to claim 5, wherein mutant p53 cells are identified by contacting the sample with a labelled antibody against mutant p53.
7. A method according to any of claims 3-6, wherein at least one antibody is labelled with a fluorescent label.
8. A method according to any preceding claim, wherein the testing is carried out using Western blotting.
9. A method according to any preceding claim, wherein the sample is a sample of cells.
10. A method according to claim 9, wherein the testing is carried out by performing a cell count.

11. A method according to claim 10, wherein the cell count is performed using multi-parameter flow cytometry.
12. A method according to claim 10, wherein the cell count is performed using scanning confocal microscopy.
13. A method according to claim 10, wherein the cell count is performed using fluorescence activated cell sorting.
14. A method according to any of claims 10-13, wherein the sample of cells is micro-dissected prior to performing the cell count, to separate normal tissue from tumour tissue.
15. A method according to claim 14, wherein normal tissue is separated from tumour tissue by contacting the sample of cells with a DNA binding dye to label aneuploid cells, and separating labelled cells from non-labelled cells.
16. A method according to claim 15, wherein the DNA binding dye is Hoechst 33258 or Chromomycin A₃ dye.
17. A method according to any of claims 10-16, wherein prior to performing the cell count, intracellular adhesion in the sample of cells is disrupted, to form a single cell suspension.
18. A method according to any preceding claim, wherein the CDK1 and CDK4 are wild-type CDK1 and CDK4.
19. A kit for the diagnosis of a cancerous or pre-cancerous state in a subject, comprising:
 - (i) a means for testing for the elevation of CDK1; and
 - (ii) a means for testing for the elevation of CDK4.

20. A kit according to claim 19, wherein the means for testing for the elevation of CDK1 comprises a labelled antibody against CDK1 and the means for testing for the elevation of CDK4 comprises a labelled antibody against CDK4.
21. A kit according to claim 20, wherein the antibody against CDK1 is sc-54 (Santa Cruz Biotechnology Inc., CA) and the antibody against CDK4 is sc-260 (Santa Cruz Biotechnology Inc., CA).
22. A kit according to any of claims 19-21, further comprising a means for identifying mutant p53 cells.
23. A kit according to claim 22, wherein the means for identifying mutant p53 cells comprises a labelled antibody against mutant p53.
24. A kit according to any of claims 20-23, wherein at least one antibody is labelled with a fluorescent label.
25. A kit according to any of claims 19-24, further comprising a DNA binding dye, for labelling aneuploid cells.
26. A kit according to claim 25, wherein the DNA binding dye is Hoechst 33258 or Chromomycin A₃ dye.
27. A kit according to any of claims 19-26, wherein the CDK1 and CDK4 are wild-type CDK1 and CDK4.
28. Use of a means for testing for the elevation of CDK1, for diagnosing cancer.
29. Use of a means for testing for the elevation of CDK4, for diagnosing cancer.

1/4

Fig.1.

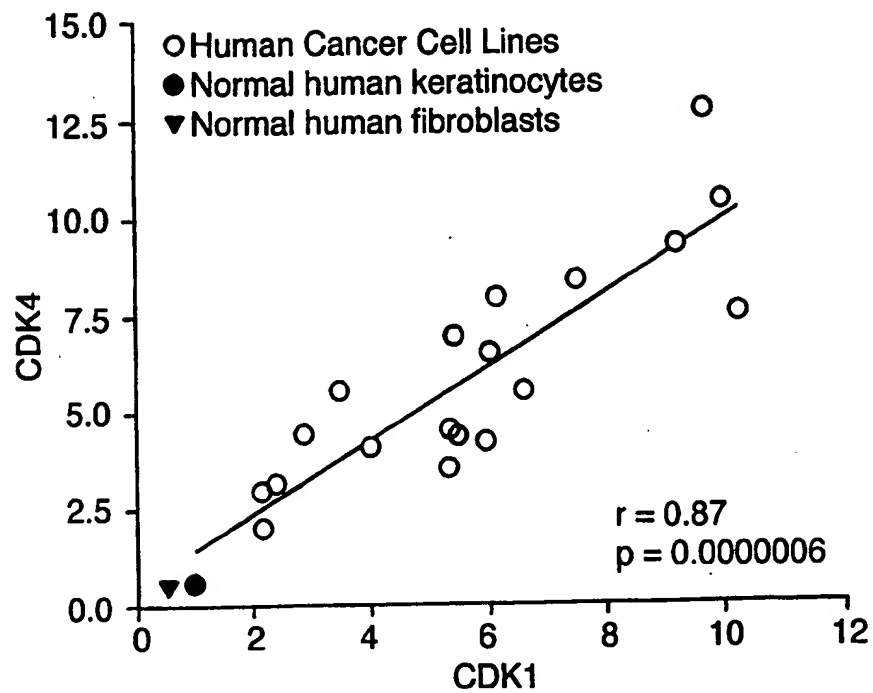


Fig.2.

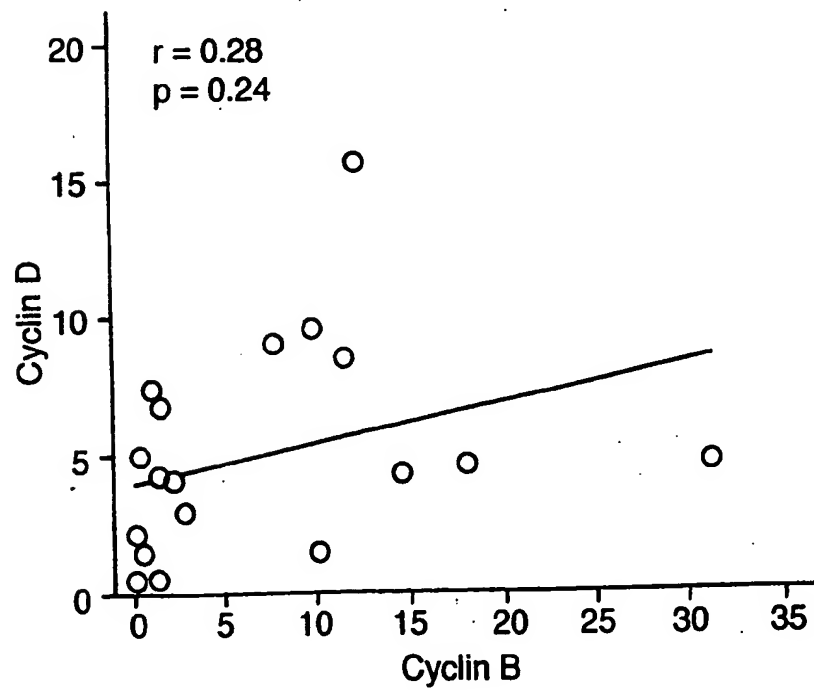
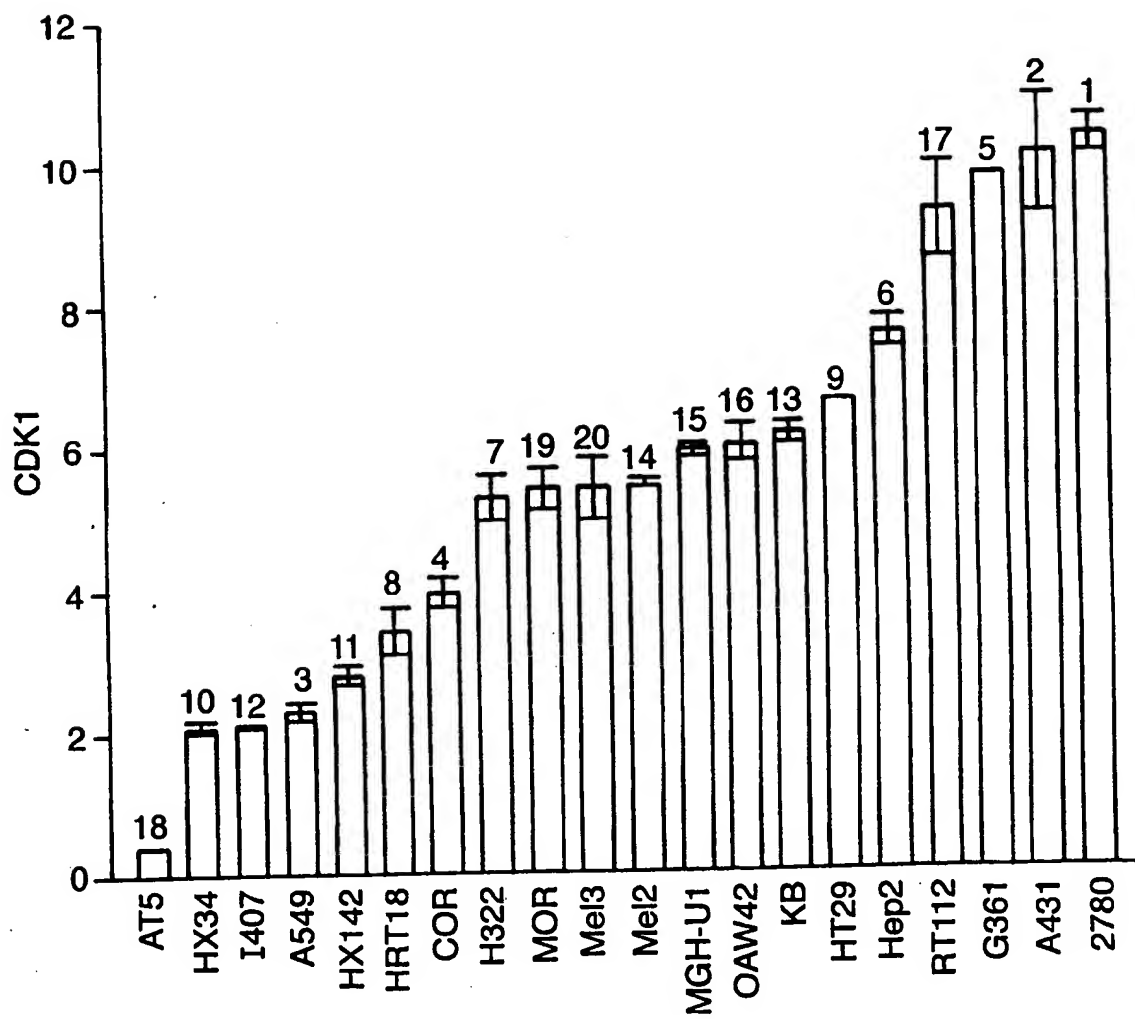
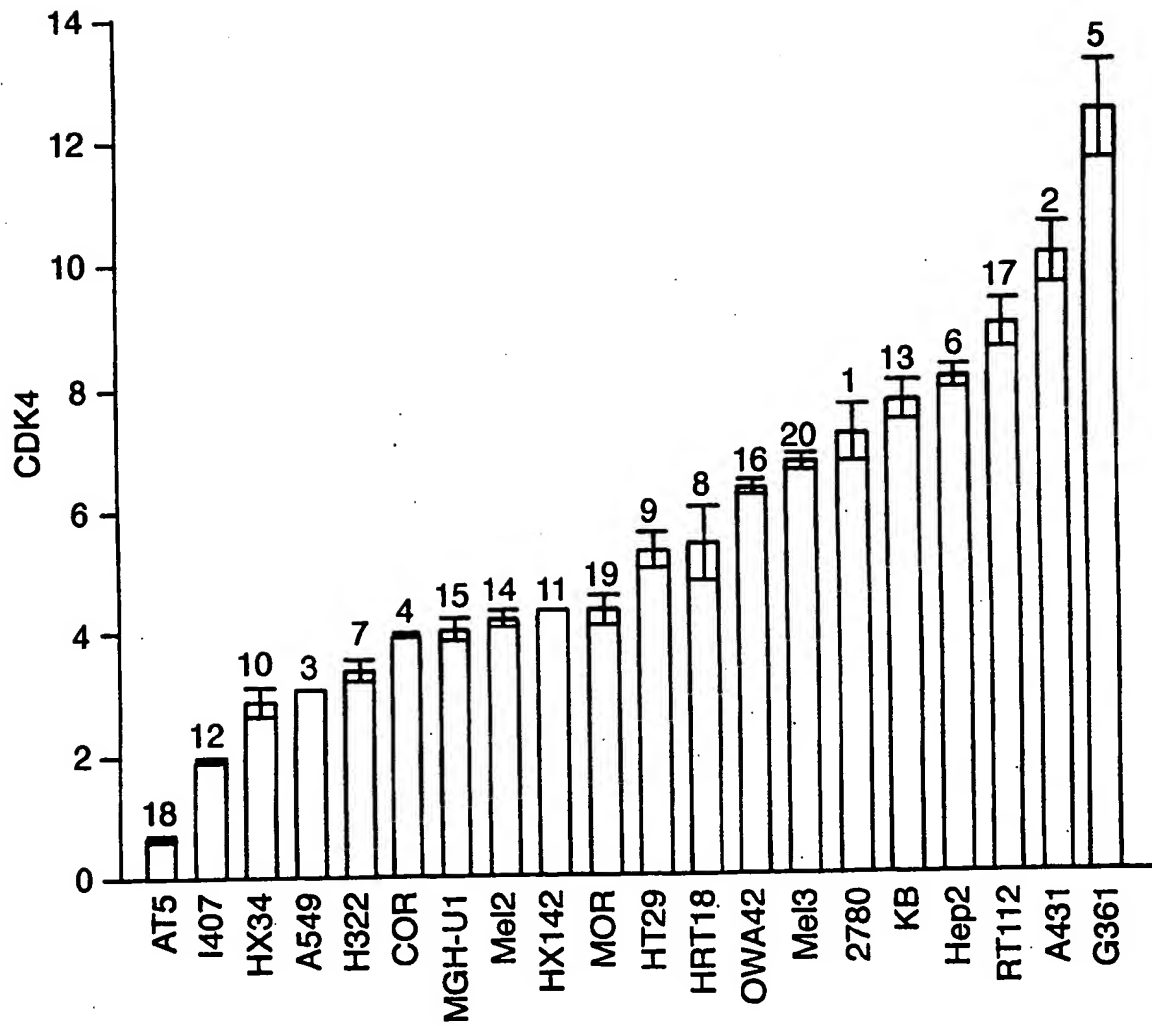


Fig.3.



3/4

Fig.4.



4/4

Fig.5.

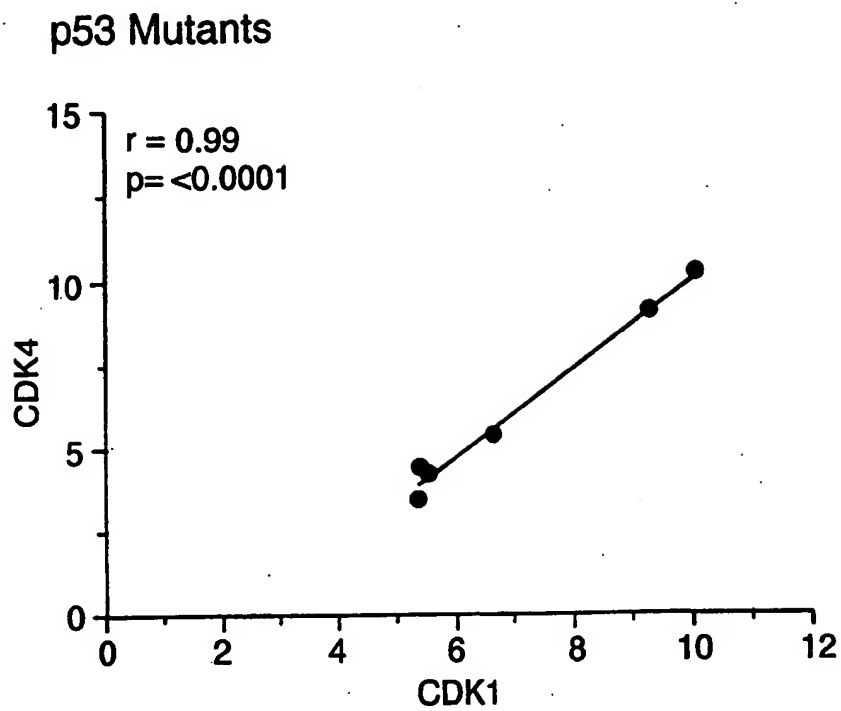


Fig.6.

